A Novel Oncolytic Herpes-Simplex-1 Virus Displays Increased Cytotoxicity in Neuroblastoma Cell Lines

Honors Research Thesis
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Abstract

Therapy using oncolytic herpes simplex-1 viruses (oHSV) involves the use of attenuated herpes simplex-1 viruses to treat and infiltrate cancer cells. However, the current viruses used have been met with resistance in some cell types due to multiple factors including lack of receptors, restricted permissivity, and stunted replication. There is a need to develop a more potent, attenuated, herpes-simplex-1 virus for oncolytic virotherapy with novel molecular pathway interactions.

The creation of oncolytic viruses capitalizes on the ability to mutate or delete key viral replication genes such as thymidine kinase, DNA polymerase, and ribonucleotide reductase. Specifically, the genes RL1 and UL39, which code for ICP34.5 and ICP6 respectively, are of pertinent interest. ICP6 is a coding unit for ribonucleotide reductase which coverts ribonucleotides into deoxyribonucleotides. Deoxyribonucleotides are important in DNA synthesis and are an abundant component of many cancerous cells. It has been shown that deleting UL39 prevents replication in non-cancerous cells which lack large reserves of deoxyribonucleotides.

In order to create a novel and more potent oHSV, the HSV-1 derived parent viruses rRp450 (RL1+/UL39-) and 17termA (RL1-/UL39+) were combined via serial passage to yield the daughter virus, Mut-3 under the strategy of directed evolution. Mut-3 was discovered to be more potent than either parent virus, displayed a unique syncytial phenotype and contained both RL1 and UL39 gene sequences. After isolating Mut-3 (RL1+/UL39+), UL39 was deleted utilizing CRISPR-Cas9 in order to ensure the safety of the virus and attenuate the product.

We hypothesize that the UL39-deleted virus, in the presence of RL1 on the potent Mut-3 backbone, will allow for greater cytotoxicity in less permissive cells. These RL1+/UL39-

mutants were isolated and purified. In the present study, we verified the viral genotype and evaluated its cytotoxic abilities. The results indicate that the increased cytotoxicity of Mut-3ΔICP6 is linked to its replicative ability and retention of the syncytial plaque phenotype in neuroblastoma cell lines.

Introduction

Neuroblastoma is the most common pediatric cancer seen in children under one years old and arise primarily from sympathetic ganglia in addition to other neural crest derivative cells [1]. Although tumors can be found in various locations throughout the body, the most common sites of primary tumors are along the posterior mediastinum, the adrenal glands, and the paravertebral ganglia [2]. For this reason, neuroblastoma is classified as a solid, extracranial tumor and accounts for 15% of pediatric cancer deaths [3].

Event-free survival of patients with metastatic disease has stalled at around 50%, despite multifaceted treatment including surgical, pharmacological, and radiological interventions [4]. These forms of therapy are often aggressive with long-lasting impacts on the health of the patient; therefore, there is a need for targeted, potent treatments that work synergistically and/or in isolation from current options.

A candidate for such treatment is oncolytic virotherapy which uses attenuated viruses to selectively infect and lyse cancerous cells. An added benefit of using oncolytic viruses is the potential to recruit the host's immune system to fight cancerous cells. Specifically, our research focuses on the use of oncolytic herpes-simplex-1 viruses (oHSV) whose genomes have a large capacity for editing and have known methodologies for attenuation [5]. Past research in our lab has shown that neuroblastoma cell lines are sensitive to oHSV [6].

Neuroblastoma is known to have varying expression levels of conventional HSV receptors [7]. There is a need to develop a more potent, attenuated, oHSV that utilizes novel techniques to increase the sensitivity and susceptibility of cancerous cells to oncolytic viruses.

Focus of the Research

The purpose of the research is to characterize the Mut-3 progeny Mut-3ΔICP6 D7-1 through the investigation of its genotype and assembly of data to determine its cytotoxicity in neuroblastoma.

Significance of the Research

Oncolytic viral therapy has proven to be a promising new pathway for selectively targeting and destroying cancerous cells. Utilizing this therapy has the potential for limiting the amount of harmful radiation and chemotherapy that a patient is exposed to, specifically in pediatric neuroblastoma patients. The age group impacted by this type of cancer is especially susceptible to the harmful effects of treatment and innovative new approaches to therapy need to be developed in order to limit the amount of harmful exposure experienced through treatment.

Oncolytic herpes-simplex virus (oHSV) therapy has proven safe in phase I pediatric trials [25] and continues to show promise in successful preclinical studies [26], [27]. These results suggest that this therapy is a viable treatment option for the pediatric population. Although the focus of this project is a pediatric model of cancer, this novel virus has far-reaching implications for treating cancer across multiple age ranges and types. Phase III adult trials utilizing oHSV therapy have also reported successful results [28], resulting in the FDA approval of the oHSV therapy *Imlygic* for the treatment of melanoma.

The creation of a novel oHSV therapy that selectively targets resistant tumor types has the potential to open up future trials and treatments to a wider range of individuals.

Definition of Key Terms

Oncolytic virotherapy- The use of viruses to selectively infect cancerous cells for the purpose of direct lysis and/or the recruitment of an immune response.

HSV-1- Herpes-simplex-1 virus.

Attenuated virus- An established virus missing key gene components that render it replication incompetent in non-cancerous cells.

Plasmid- A circular piece of extra-chromosomal DNA that can be altered to include genes of interest. Plasmids can be used in transfection procedures to insert DNA sequences into a genome.

Directed evolution- A methodology used to artificially simulate natural selection to guide (in this case) a virus to obtain new characteristics.

ICP34.5- A neurovirulence protein encoded by the RL1 gene that acts in viral replication processes.

ICP6- A protein encoded by the UL39 gene that works as a part of viral ribonucleotide reductase function.

CRISPR-Cas9- A novel gene editing system that uses guide RNAs to attract the Cas9 protein to a specific genetic sequence where it makes a double stranded cut in DNA. This cut can be repaired through homologous recombination by supplying a repair construct.

rRp450- An established oncolytic HSV-1 being used in Phase 1 clinical trials for the treatment of liver cancer. rRp450 expresses the rat transgene CYP2B1, an enzyme that induces activation of the prodrug form of the chemotherapy cyclophosphamide [8]. Genotype: UL39-/RL1+.

17TermA- An established oncolytic HSV-1 being used in pre-clinical tests. Genotype: UL39+/RL1- [29].

Mut-3- The parental HSV-1 created using directed evolution from rRp450 and 17TermA. Mut-3 is replication competent in non-replicative normal cells and cancerous cells due to its genotype: Genotype: UL39+/RL1+.

Mut-3ΔICP6 D7-1- A daughter HSV-1 strain of Mut-3 created using CRISPR-Cas9 editing to delete UL39, the gene encoding ICP6. Mut-3ΔICP6 D7-1 is replication incompetent in non-replicative normal cells and replication competent in cancerous cells due to its genotype: UL39-/RL1+.

Background

Projections estimate that there will be up to 27.5 million new cancer cases worldwide by the year 2040 and is currently the second leading cause of death globally [9]. 15,000 of reported cases are in pediatric patients within the United States [10]. These patients undergo a variety of interventions, depending on cancer type, that can include surgical resection, radiotherapy, chemotherapy, and bone-marrow transplant. Pediatric cancer survivors are subject to severe long-term side effects including neurocognitive discrepancies, and the potential for therapy-related secondary cancers [11].

Neuroblastoma is the most common extracranial solid tumor in pediatric patients and can be found throughout the body, most commonly on or around the adrenal glands [3]. A strategy being actively researched is the use of herpes-simplex-1 viruses (oHSVs) to selectively infect and destroy cancerous cells; however, in cells lines such as NB88R2, a primary Epstein-Barr virus (EBV)-infected lymphocyte cell culture from a neuroblastoma patient, low expression of HSV-1 receptors on the cell surface can be a barrier of oHSV infection [12]. Since expression levels of HSV-1 receptors vary across cancerous cell types, a way to bypass the receptor barrier was desired [13] [14] [15]. The strategy of directed evolution, involving artificially simulating natural selection, was employed for this purpose [16].

The previously described parental oHSVs rRp450, an ICP6-null virus [17], and 17TermA, an ICP34.5-null virus [18], were co-infected onto NB88R2 cells. Alone, each virus cannot infect the cell line, but under the pressures of directed evolution, the viruses recombined. The result was Mut-3, a wild-type-like virus with a syncytial plaque phenotype of area larger than those produced by either parental virus. Additionally, Mut-3 was found to be more cytotoxic than either parental virus. Despite Mut-3's superior cytotoxicity, it is an unattenuated virus containing

both UL39 and RL1 genes that indiscriminately infects both cancerous and non-replicative normal cells. The deletion of UL39 has been shown to attenuate HSVs, causing them to become selective for infection of cancerous cells [19]. The potency of the Mut-3 construct provided rationale for the investigation into the creation and testing of an attenuated Mut-3 progeny, Mut-3ΔICP6.

Research Aims and Related Hypotheses

Aim 1: To attenuate wild-type-like Mut-3 through the deletion of ICP6. Mut-3ΔICP6 will be created through the excision of UL39 using CRISPR-Cas9. A GFP reporter construct, supplied via plasmid, will be inserted in place of the gene. We hypothesize that Mut-3 will be attenuated with the deletion of UL39 and subsequent loss of the expression of ICP6.

Aim 2: To purify and validate the genotype of an ICP6-null progeny of Mut-3, as well as define its safety profile. Purification will be achieved via serial plaque purification. qPCR validation will be used to ensure the deletion of UL39 from the viral genome. Cytotoxicity assays will be performed on non-replicative normal cells to assess selectivity of the virus compared to Mut-3. We hypothesize that Mut-3ΔICP6 exhibits increased potency compared to other oHSV options while retaining safety in non-replicative normal cells.

Aim 3: To explore the therapeutic abilities of Mut-3ΔICP6 in the treatment of murine and human neuroblastoma cells lines in comparison to present oHSVs. Mut-3ΔICP6 will be compared with Mut-3 and rRp450. These viruses will be used to evaluate the cytotoxicity and replication ability of Mut-3ΔICP6 in murine and human neuroblastoma cell lines. We hypothesize that Mut-3ΔICP6 will target cancerous cells more effectively than other ICP6-null oHSV vectors. Mut-3ΔICP6 will retain the superior killing ability of Mut-3, but with precise selectivity.

Methodology

This research study will be completed in the research laboratory of Dr. Timothy Cripe, MD, PhD in the Center for Childhood Cancer and Blood Diseases in the Research Institute at Nationwide Children's Hospital. The Center for Childhood Cancer and Blood Diseases is affiliated with the James Comprehensive Cancer Center at The Ohio State University Wexner Medical Center. This research is being completed under the supervision of Dr. Pin-Yi Wang, PhD. The technical methodology of each aim is detailed below.

Aim 1: Attenuation of Mut-3. The plasmid PCK #1061 (supplied by Dr. Kevin Cassady, NCH) was utilized to create a repair plasmid for use in conjunction with CRISPR-Cas9. Plasmid PCK #1061 contains the full coding region of UL39 and is flanked by UL37, UL38, and UL40 mimicking the HSV genome. This construct composition allows for utilization as the repair plasmid in the CRISPR-Cas9 reaction following excision of UL39 from PCK #1061. PCK #1061 was digested with restriction enzymes BglII and AgeI for 2 hours at 37ºC for the purpose of linearization and exclusion of UL39. The sample was run on a 1% EtBr agarose gel at 100V for 30 minutes. The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). Next, PCR primers were designed using the InFusion Primer Design Tool (Clontech, Mountain View, CA, USA) for the purpose of amplifying an EGFP cassette with 15 base pair extensions that were complementary to the ends of the linearized vector. Plasmid PCK #1039 (supplied by Dr. Kevin Cassady, NCH), containing the EGFP cassette to serve as a template, underwent PCR amplification utilizing the aforementioned primers. The sample was run at 98°C for 30 seconds, 35 cycles of 98º C for 10 seconds, 53º C for 15 seconds, and 72º C for 20 seconds followed by 5 minutes at 70º C. The sample was run on a 1% EtBr agarose gel at

100V for 30 minutes. The PCR product was purified using the QIAquick PCR Purification Kit. An InFusion cloning reaction using an In-Fusion HD Cloning Kit (Clontech, Mountain View, CA, USA) was performed using the purified, linearized vector and the purified, amplified EGFP cassette. The reaction mixture was subsequently used to transform Stellar competent cells according to the guidelines of the In-Fusion HD Cloning Kit. Following transformation and plating on LB/Amp agar plates, six colonies were chosen for plasmid purification using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA). Each clone was then digested with restriction enzymes BglII, AgeI, PacI, and BamHI for 2 hours at 37ºC for the purpose of confirming the repair plasmid construct. The sample was run on a 1% EtBr agarose gel at 100V for 30 minutes. The baby hamster kidney (BHK-21) cell line was purchased from ATCC and grown up in a 12-well plate at a density of 1.8e6 cells per plate. The transfection mixture was composed of a 1:2 ratio of Cas9/gRNA to repair plasmid, 2.5uL of P3000 (ThermoFisher Scientific, Waltham, Massachusetts, USA), 2.5uL of Lipofectamine (ThermoFisher Scientific, Waltham, Massachusetts, USA), and 50uL of OPTI-MEM (ThermoFisher Scientific, Waltham, Massachusetts, USA). This reaction was incubated for 15 minutes at room temperature. The confluent BHK cells were washed with phosphate buffer solution (PBS) and 400uL of OPTI-MEM was added in addition to the transfection mixture. The cells were incubated for 5 hours at 37ºC. Following incubation, Mut-3 at MOI=0.05 (multiplicity of infection) in a volume of 1uL, was added to all the wells. Lysis and cytopathic effect (CPE) were assessed daily and lysate was stored at -80ºC.

Aim 2: Purification, genotype validation, and safety in non-replicative normal cells of Mut-3ΔICP6 D7-1. Both BHK-21 cells and Vero cells (ATCC), from African green monkey kidney, were used to serial plaque purify Mut-3ΔICP6. Cells were assessed under a fluorescent scope

(ThermoFisher Scientific, Waltham, Massachusetts, USA). Clones were considered purified when all plaques in a well were syncytial and GFP positive. Purified clone Mut-3ΔICP6 D7-1 was used to perform a viral preparation. In a 6 well plate of 2e6 BHK cells, individual wells were infected with Mut-3ΔICP6 D7-1, rRp450, or Mut-3 at MOI=0.1. Genomic DNA (gDNA) was isolated from these cells following 24 hours of incubation at 37ºC using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). The gDNA was then used to perform quantitative polymerase chain reaction (qPCR) with Power SYBR Green PCR Master Mix (Life Technologies, Carlsbad, CA, USA) an ICP6 primer [20], and GFP primer (Integrated DNA Technologies, Coralville, IA, USA). The reaction was performed using Applied Biosystems 7900 Real-Time PCR system (Life Technologies). The samples were run at 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds followed by a standard dissociation stage to determine the melting temperature of each amplification product. The resulting detection was presented as expression fold relative to an internal control, the HSV thymidine kinase gene (TK). Non-replicative normal cells were modeled using normal human foreskin keratinocytes (HFKs) obtained from normal newborns following circumcision (Wells, Cincinnati Children's Research Foundation, Cincinnati, OH) [17]. HFK cells were grown in EpiLife Media (Cascade Biologics) supplemented with human growth supplement keratinocyte according to the manufacturer's instructions. Differentiated HFKs were obtained by the addition of 10% FBS and 1 mmol/l $CaCl_2$ [21]. HFK survival was measured by MTS assay. Undifferentiated HFKs were seeded into a 96-well plate at a density of 2000 cells per well and cultured overnight. The cultures were then infected with Mut-3 or Mut-3ΔICP6 D7-1 at MOIs of 0.0004, 0.004, 0.04, 0.4, and 4. The cell survival was determined after 3 days compared to uninfected controls. This same process was repeated to produce differentiated cells, but

differentiation was induced 24 hours post-seeding and was allowed to continue for 48 hours before infection. The cell survival was determined after 4 days compared to uninfected controls.

Aim 3: Cell survival/MTS and virus production. A panel of murine (Neuro-2a and 975A2) and human (CHP-134 and SK-N-AS) neuroblastoma cancer cell lines was used for the experiments in this study. The cell lines were obtained from the Pediatric Preclinical Testing Program (PPTP) database (Houghton, NCH) and from ATCC. The human cell lines were confirmed with STR. Both murine and human cell lines were mycoplasma negative. All cell lines were maintained in the media recipes recommended by ATCC. NB cell survival was measured by MTS assay. Individual cell lines were seeded into a 96-well plate at a density of 4000 cells per well and cultured overnight. The cultures were then infected with Mut-3, rRp450, or Mut-3ΔICP6 D7-1 at MOIs of 0.001, 0.01, 0.1, 1, and 10. The cell survival was determined after 3 days compared to uninfected controls. Viral production assay was performed on 975A2 and SK-N-AS cells. The cells were plated in 12-well dishes at 1.5e5 cells per well in triplicate, incubated at 37ºC for 24 hours, and infected with either rRp450 or Mut-3ΔICP6 D7-1 at MOI=0.1 for SK-N-AS and MOI=0.5 for 975A2. Plates were gently shaken every 20 minutes for 2 hours. At 2, 24, 48, and 72 hours post infection, the cells and supernatants were harvested. These samples were freeze-thawed three times, diluted, and titered by plaque assay on Vero cells as previously described [22].

Results

CRISPR-Cas9 can be utilized to attenuate Mut-3 through the deletion of UL39. CRISPR-Cas9 has been shown to be an efficient method for viral editing in the creation of new oncolytic viruses [23]. The use of a GFP reporter sequence allowed for the confirmation of two processes: the successful integration of the GFP cassette into Mut-3 (construct shown in Fig. 1) in place of UL39, and the ability to visualize infection of target cells (Fig. 2).

The genotype of Mut-3ΔICP6 is UL39-null and GFP-positive. Quantitative polymerase chain reaction (qPCR) evaluated the expression of ICP6 and GFP in Mut-3, rRp450, and Mut-3ΔICP6 D7-1 relative to the HSV thymidine kinase (TK) internal control. Mut-3 served as an ICP6 positive control, whereas rRp450 served as an ICP6 negative control. The results show ICP6 expression in Mut-3 approximately 2.4-fold expression relative to TK, whereas rRp450 and Mut-3ΔICP6 D7-1 expression is negligible (Fig. 3). The results show GFP expression in Mut-3ΔICP6 D7-1 approximately 0.8-fold expression relative to TK, whereas rRp450 and Mut-3 expression is negligible (Fig. 4).

Mut-3ΔICP6 D7-1 potency is significantly attenuated in HFK cells. To assess the attenuation of Mut-3ΔICP6 D7-1, we measured the survival of primary human foreskin keratinocytes (HFKs) when exposed to Mut-3ΔICP6 D7-1 relative to its parental, wild-type-like virus, Mut-3. In undifferentiated, dividing HFKs, Mut-3 and Mut-3ΔICP6 D7-1 act similarly in terms of cytotoxicity (Fig. 5). In differentiated, quiescent HFKs, Mut-3 showed significant cytotoxicity, even at low MOIs. By contrast, the cytotoxicity of Mut-3ΔICP6 D7-1 was severely attenuated (Fig. 6). These data suggest that Mut-3ΔICP6 D7-1 is more attenuated than Mut-3.

Mut-3ΔICP6 D7-1 displays increased cytotoxicity in murine and human neuroblastoma cell lines. Mut-3ΔICP6 D7-1 is effective in neuroblastoma cell line killing (4 cell lines shown). hNB cell line SK-N-AS showed significant superiority of Mut-3ΔICP6 D7-1 over Mut-3 at MOI 1, but overall, all three viruses were highly cytotoxic to this cell line (Fig. 7). hNB cell line CHP-134 showed significant superiority of Mut-3ΔICP6 D7-1 over rRp450 beginning at MOI 0.1 (Fig. 8). mNB cell line 975A2 showed significant superiority of Mut-3ΔICP6 D7-1 over rRp450 and Mut-3 beginning at MOI 1 (Fig. 9). mNB cell line Neuro-2a showed significant superiority of Mut-3ΔICP6 D7-1 over rRp450 and Mut-3 beginning at MOI 0.1 (Fig. 10).

Mut-3ΔICP6 D7-1's increased cytotoxicity appears to be correlated with superior replicative ability in neuroblastoma cell lines. In order to investigate a potential mechanism of the increased cytotoxicity of Mut-3ΔICP6 D7-1 seen in MTS assay, viral production assay was performed using mNB and hNB cells. At 48 hours post-viral infection, Mut-3ΔICP6 D7-1 showed a statistically significant ($p=0.0497$) increase in viral production compared to rRp450 in murine 975A2 cells. Statistical significance increased ($p=0.0037$) at 72 hours post-viral infection between Mut-3ΔICP6 D7-1 and rRp450 (Fig. 11).

From 2 hours to 48 hours post-viral infection, Mut-3ΔICP6 D7-1 showed a statistically significant (respectively, $p=0.0092$, 0.0441, 0.0460) increase in viral production compared to rRp450 in human SK-N-AS cells. Statistical significance was lost at 72 hours post-viral infection between Mut-3ΔICP6 D7-1 and rRp450 production (Fig. 12). Together with the results in Figures 7-10, these results suggest that the increased cytotoxicity of Mut-3ΔICP6 is correlated to its replicative ability in neuroblastoma cells.

Figures

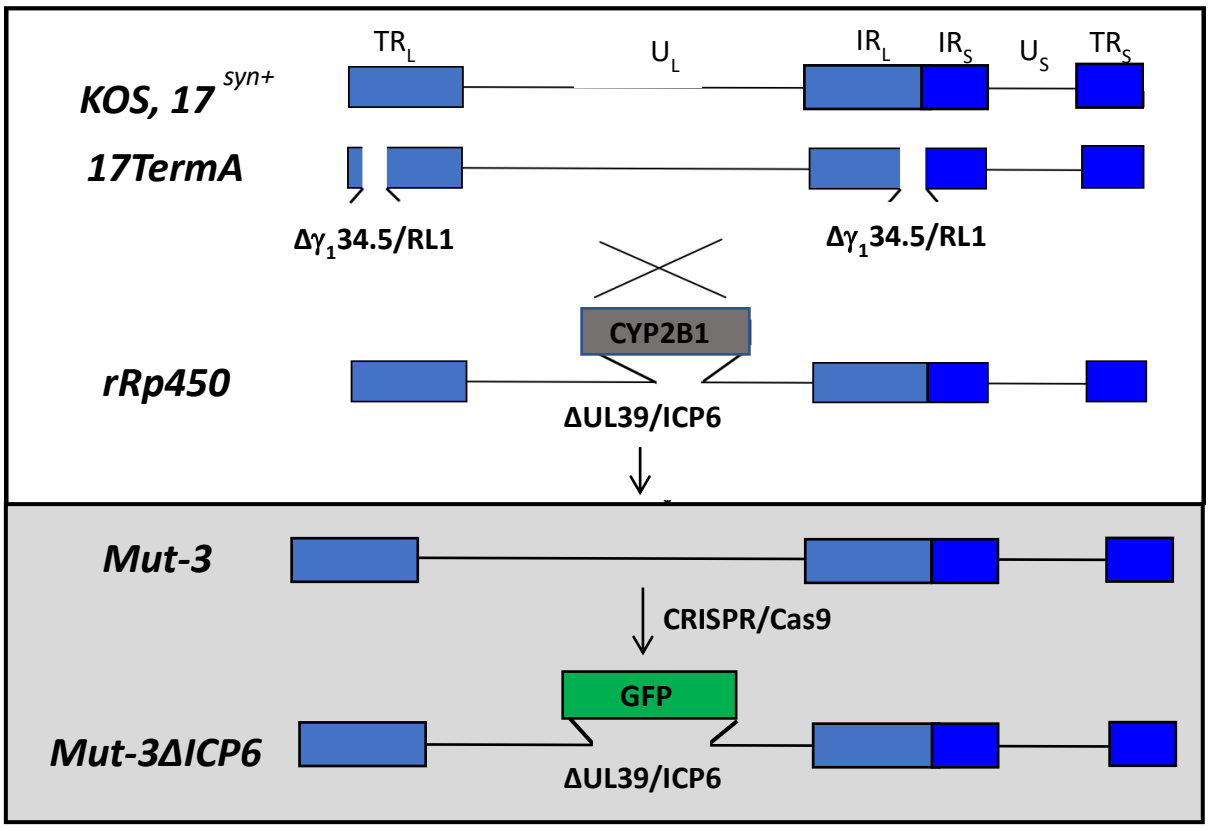

Figure 1: Schematic representation of the 17TermA, rRp450, Mut-3, and Mut-3ΔICP6 genomes.

Attenuation of Mut-3 is phenotypically confirmed by the presence of GFP and retention of syncytia

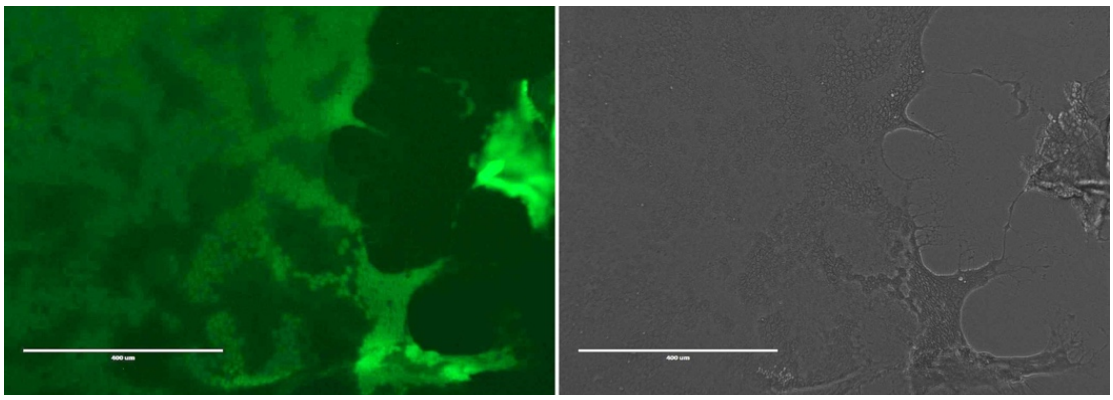

Figure 2: Infection of BHK cells with Mut-3ΔICP6 D7-1. Successful UL39 knock-out of Mut-3 is evidenced by the presence of GFP fluorescence. Positive infection is confirmed by the presence of GFP fluorescence and the syncytial plaque phenotype.

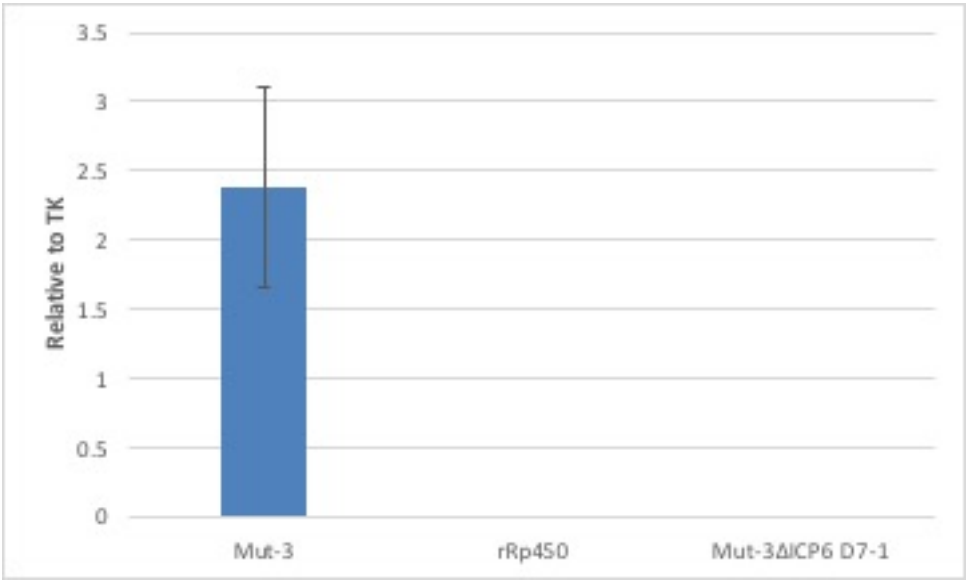

Figure 3: qPCR analysis for ICP6 content in gDNA. Data are presented relative to TK.

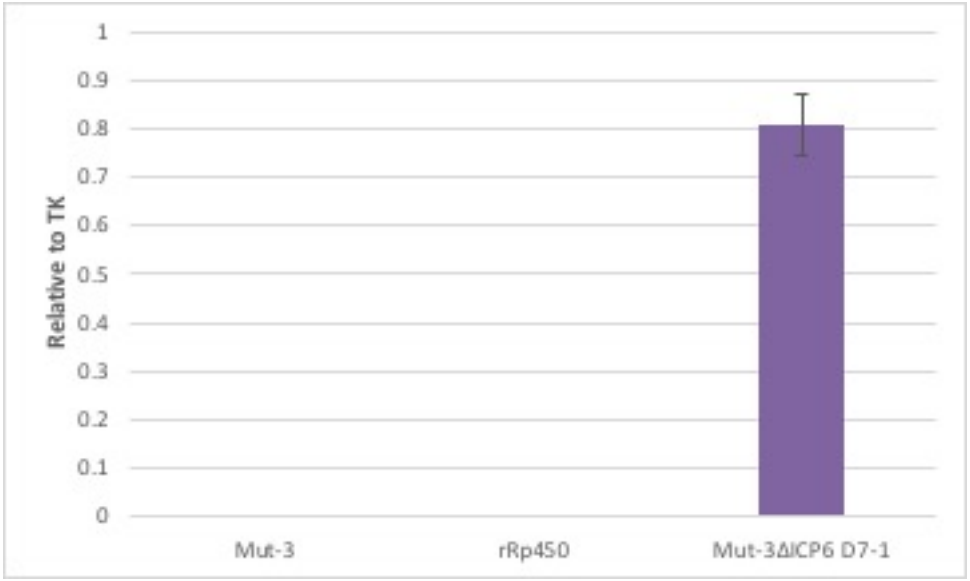

Figure 4: qPCR analysis for GFP content in gDNA. Data are presented relative to TK.

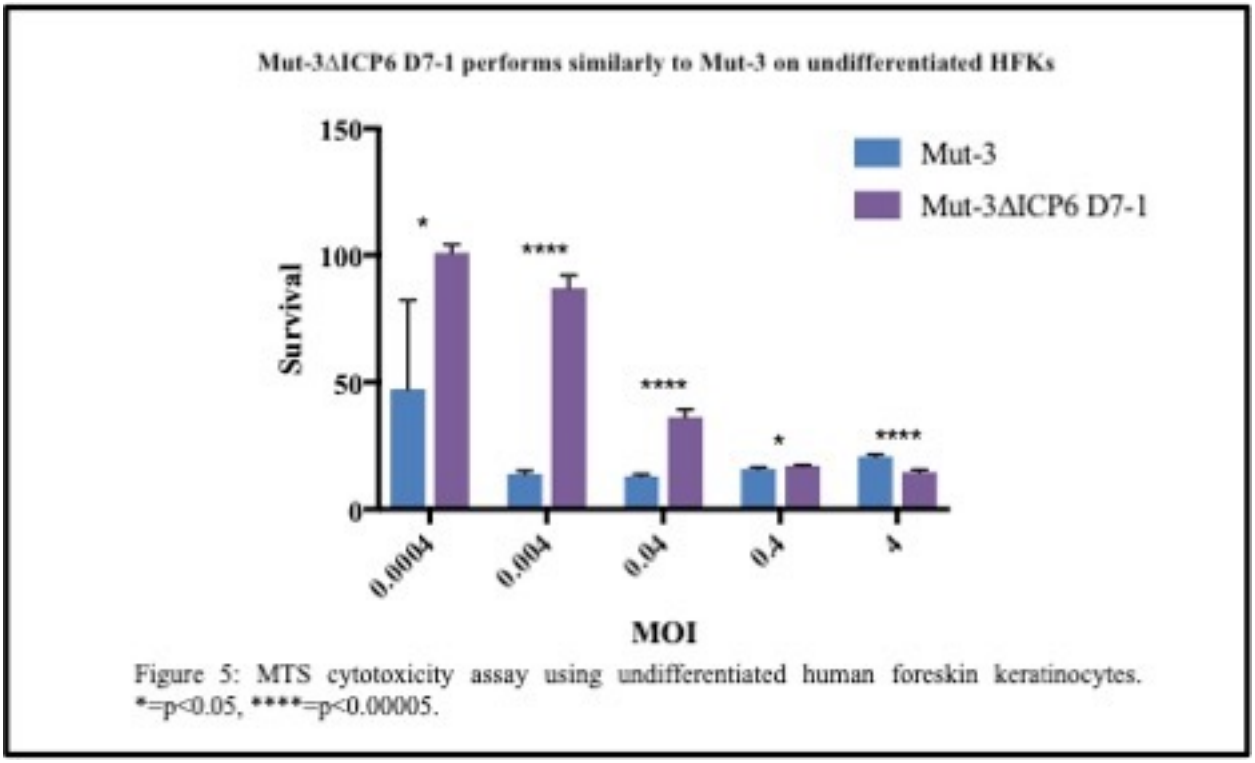

Figure 5: MTS cytotoxicity assay using undifferentiated human foreskin keratinocytes. *=p<0.05, ****=p<0.00005.

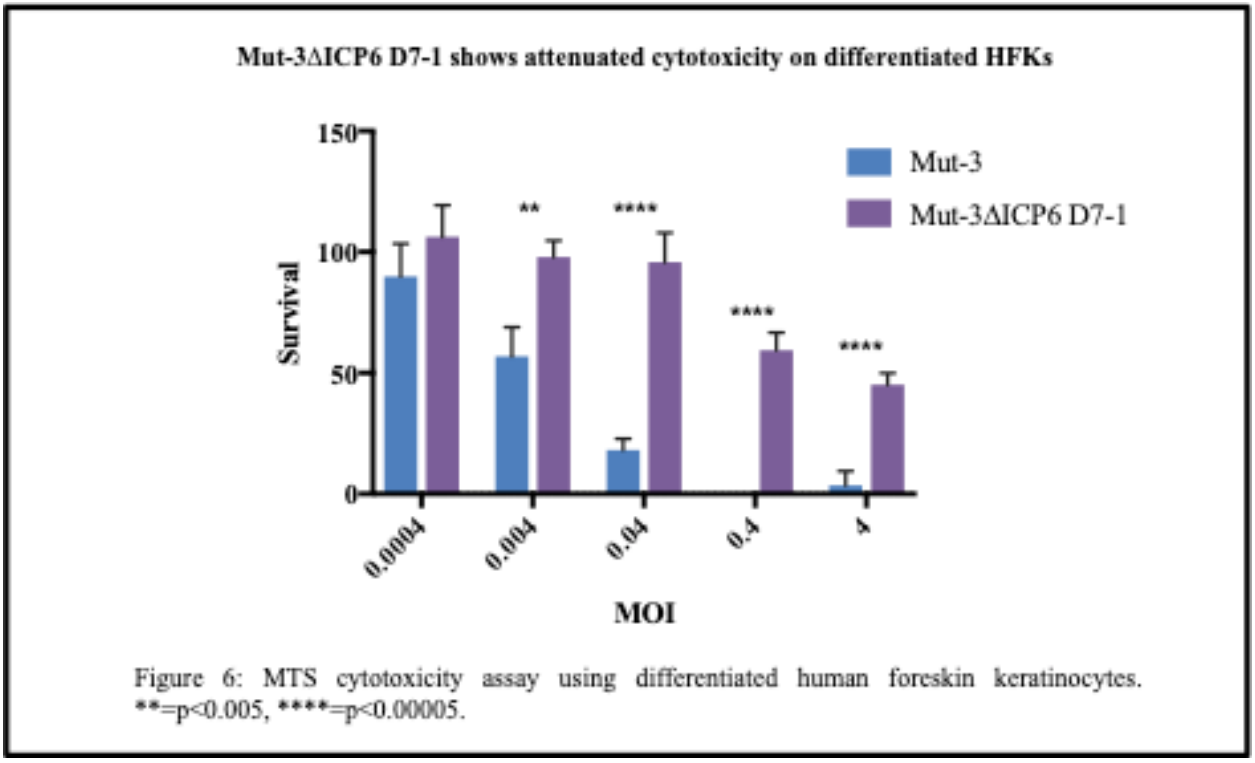

Figure 6: MTS cytotoxicity assay using differentiated human foreskin keratinocytes. **=p<0.005, ****=p<0.00005.

Mut-3ΔICP6 D7-1 does not show significant cytotoxicity over Mut-3 and rRp450 in SK-N-AS

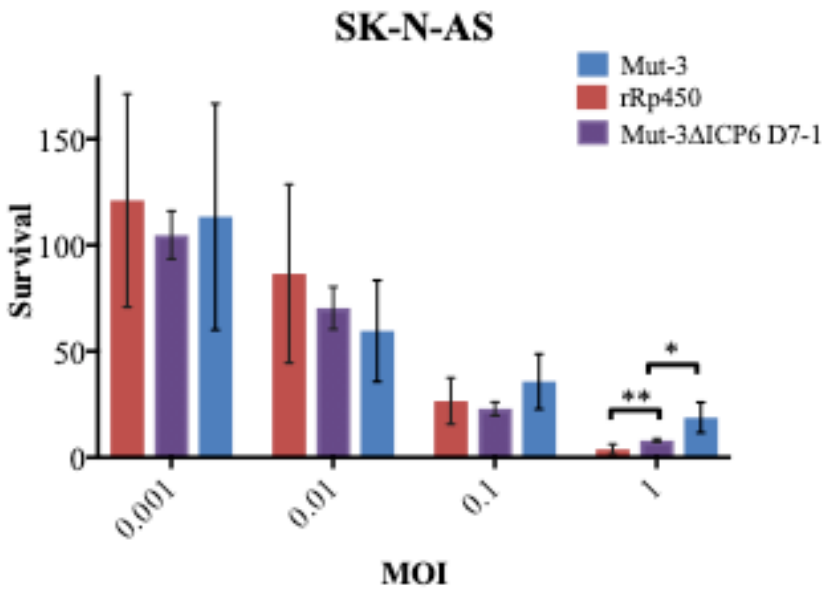

Figure 7: MTS cytotoxicity assay using hNB line SK-N-AS. **=p<0.005, *=p<0.05.

Mut-3ΔICP6 D7-1 does show significant cytotoxicity over Mut-3 and rRp450 in CHP-134

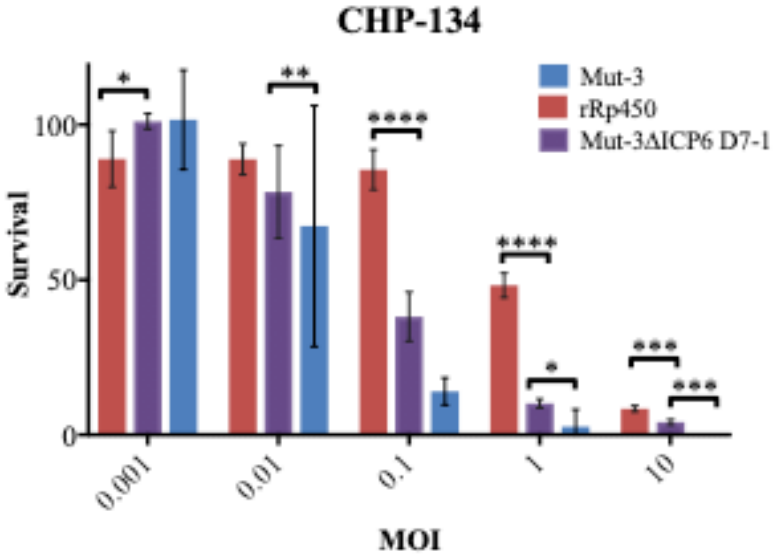

Figure 8: MTS cytotoxicity assay using hNB line CHP-134. ****=p<0.00005, ***=p<0.0005, **=p<0.005, *=p<0.05.

Mut-3ΔICP6 D7-1 does show significant cytotoxicity over Mut-3 and rRp450 in 975A2

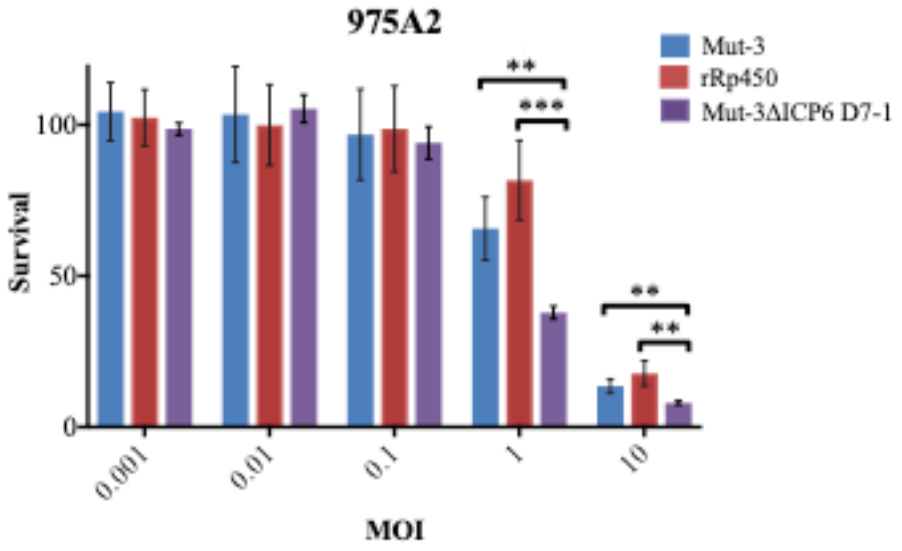

Figure 9: MTS cytotoxicity assay using mNB line 975A2. ***=p<0.0005, **=p<0.005.

Mut-3ΔICP6 D7-1 does show significant cytotoxicity over Mut-3 and rRp450 in Neuro-2a

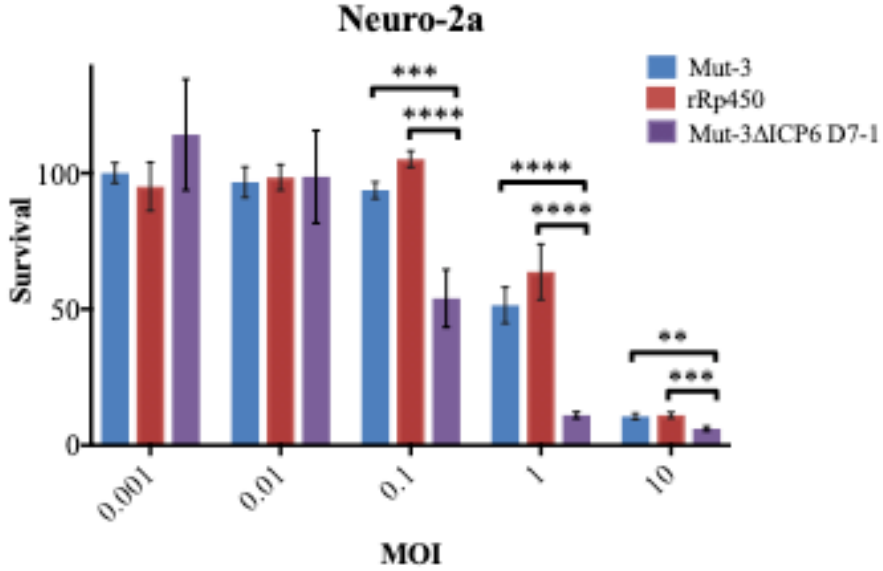

Figure 10: MTS cytotoxicity assay using mNB line Neuro-2a. ****=p<0.00005, ***=p<0.0005, **=p<0.005.

Mut-3ΔICP6 D7-1 shows enhanced viral production over rRp450 at 72 hours post-infection

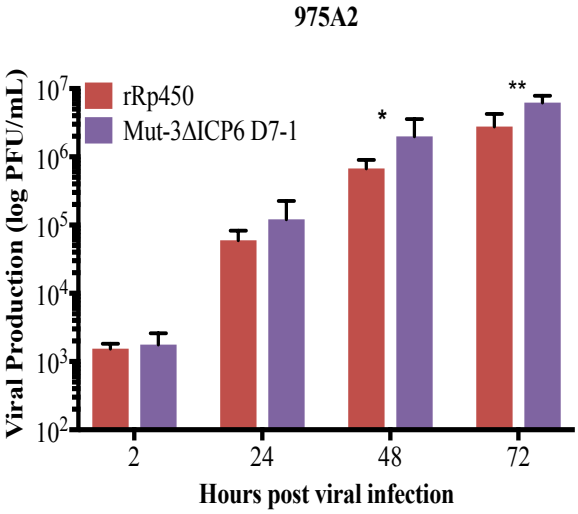

Figure 11: Viral replication assay using mNB line 975A2. **=p<0.005, *=p<0.05.

Mut-3ΔICP6 D7-1 shows comparable viral production to rRp450 at 72 hours post-infection

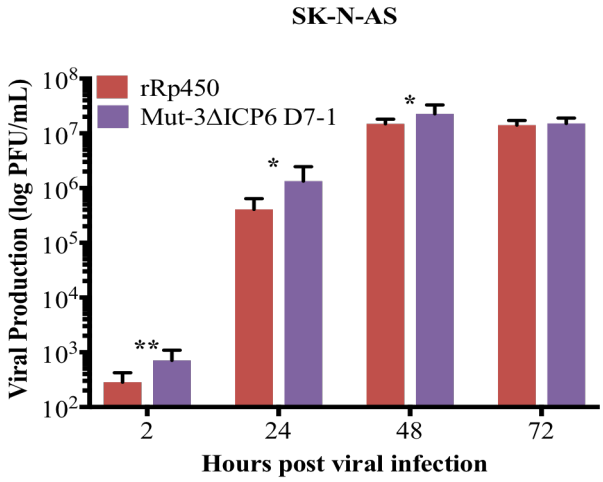

Figure 12: Viral replication assay using hNB line SK-N-AS. **=p<0.005, *=p<0.05.

Discussion

Oncolytic virotherapy is a promising approach to cancer treatment because it can cause the direct lysis of cancerous cells in addition for the potential to induce an immune response against these cells [24]. Developing highly specific, attenuated viruses that retain cytotoxicity in target cells is vital to this technique.

This research characterized the Mut-3 progeny Mut-3ΔICP6 D7-1 by its genotype, cytotoxicity, and replicative ability in neuroblastoma cell lines. The use of CRISPR-Cas9 for the attenuation of the wild-type-like virus Mut-3 was shown to be an appropriate methodology. We confirmed our initial hypothesis that attenuation of Mut-3 could be achieved with the deletion of UL39 and subsequent loss of ICP6.

Although this technique was effective, it was determined that the construct of the repair plasmid is crucial for successful UL39 knock-out in Mut-3. Initially, we attempted to use the plasmid PCK #1039 which has an EGFP cassette disrupting the UL39 gene. It was discovered that the plasmid was recombining in the presence of Mut-3. This caused EGFP to be kicked out, resulting in UL39 recovery. We revised our strategy and utilized PCK #1061 to replace almost all of UL39 with EGFP, preventing the potential for UL39 recovery. Further research could include using this methodology to create additional versions of Mut-3, with careful consideration to plasmid design.

Using quantitative polymerase chain reaction (qPCR), the ICP6-null status of Mut-3ΔICP6 D7-1 was confirmed. ICP6 detection was negligible and comparable to the established ICP6-null rRp450 [17]. This methodology also confirmed the presence of GFP in the genome, indicating that the repair plasmid successfully permitted the incorporation of GFP in place of UL39 within the Mut-3 genome.

We next confirmed our hypothesis that Mut-3ΔICP6 D7-1 has attenuated cytotoxicity in non-replicative normal human cells. Using differentiated human foreskin keratinocytes as a model of non-replicative normal human cells, the wild-type-like Mut-3 was compared to attenuated Mut-3ΔICP6 D7-1. Testing resulted in the confirmation of our hypothesis that Mut-3ΔICP6 retains safety in non-replicative normal human cells. Mut-3 showed significant cytotoxicity against these cells at both high and low MOIs, whereas the cytotoxicity of Mut-3ΔICP6 D7-1 was severely attenuated.

To evaluate the cytotoxicity of Mut-3ΔICP6 D7-1 in neuroblastoma, both murine and human neuroblastoma cell lines were used. Human cells were used to model patients who present in the clinic, whereas murine cells represented the mouse models employed to test oHSV *in vivo*. In murine cell lines 975A2 and Neuro-2a, Mut-3ΔICP6 D7-1 showed significant cytotoxicity over Mut-3 and rRp450 starting at MOI 0.1 for Neuro-2a and MOI 1 for 975A2. In human cell line SK-N-AS, Mut-3ΔICP6 D7-1 performed similarly to Mut-3 and rRp450 in terms of cytotoxicity. In human cell line CHP-134, Mut-3ΔICP6 D7-1 showed significant cytotoxicity over rRp450 beginning at MOI 0.1. The human results prompted further investigation into a mechanism behind Mut-3ΔICP6 D7-1's cytotoxicity strategy, and, importantly, showed that human neuroblastoma cells are sensitive to this virus. The murine results provided a rationale to advance into *in vivo* toxicity studies which are currently ongoing. *In vivo* results, as well as continued *in vitro* assays of various types of neuroblastoma cell lines will assist in growing our understanding of Mut-3ΔICP6 D7-1's cytotoxic abilities and range.

To correlate Mut-3ΔICP6 D7-1 cytotoxicity with a mechanism, viral production assays were performed using SK-N-AS and 975A2. Most pertinent were the samples collected at 72 hours post viral infection as this is the same time point at which the MTS assays were read.

Statistical significance in Mut-3ΔICP6 D7-1 viral production over rRp450 was seen in 975A2 at 72 hours. This correlates with Mut-3ΔICP6 D7-1's statistically significant cytotoxicity over rRp450 seen in MTS assay. Additionally, statistical significance in Mut-3ΔICP6 D7-1 viral production over rRp450 was not seen in SK-N-AS at 72 hours. This correlates with the lack of Mut-3ΔICP6 D7-1 statistical significance over rRp450 seen in MTS assay. Cumulatively, these results imply that Mut-3ΔICP6 D7-1's cytotoxicity stems from its replicative ability, likely linked to its retention of the parental syncytial plaque phenotype.

The present research found Mut-3ΔICP6 D7-1 to be an attenuated, cytotoxic, and selective progeny of Mut-3. Mut-3ΔICP6 D7-1 shows promise as a potent, cancer-cell specific viral therapeutic. The current findings will continue to be investigated both *in vitro* and *in vivo* with the hopes of bringing this form of oHSV into clinical trials and improve the outcomes of children with neuroblastoma.

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